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# Chapter 9

## Modeling Risk Factors and Confounding Effects in Stroke

Barry McColl, David Howells, Nancy Rothwell, and Adam Denes

### Abstract

Most research to date has used experimental models in rodents which fail to mimic the underlying causes of stroke in patients or the primary confounding factors. Available data indicate that factors such as atherosclerosis, hypertension, obesity, diabetes, age, and inflammation have a major influence on outcome. These findings suggest that we need to rethink the preclinical data that are required before selection of candidate interventions for clinical trials in stroke.

**Key words:** Atherosclerosis, Hypertension, Obesity, Diabetes, Age, Inflammation

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### 1. Introduction

Animals have been used successfully to study the underlying mechanisms of diverse diseases and to successfully develop new treatments. In many cases, the diseases in question are induced through a means which differs significantly from the clinical course and causes (e.g., implanted tumors in young and otherwise healthy animals) mechanical or pharmacological induction of hypertension and cardiac disease, and pharmacological induction of diabetes. Some have used spontaneous mutations that result in the disease of interest (e.g., genetically obese animals), mutagenesis to cause a pathology (e.g., deafness), or specific genetic manipulation to induce disease (e.g., to cause mutations known to occur in Alzheimer's). All of these have been very helpful in understanding disease pathology and in the development and testing of new medicines.

The situation in experimental studies on stroke is quite different because the animal models we have used have revealed much knowledge of underlying pathological mechanisms, but have (with the notable exception of tPA) failed to deliver new medicines.

Thus, after many promising preclinical outcomes, we have numerous failed clinical trials in stroke. Many of these can be explained on the basis of inadequate preclinical data, poor clinical trial design, or a mismatch between the preclinical findings and the clinical studies; some failures remain inexplicable. This has caused a serious reanalysis of our experimental approaches to stroke, including the rigor of the experimental work and the relevance of commonly used methods (1–5).

To date, the vast majority of experimental studies on stroke have used young male rodents, with no confounding comorbidities, no parallel treatments, and no consequences of stroke. Furthermore, few studies have used approaches which mimic the clinical causes of stroke to induce cerebral ischemia. Indeed most have employed mechanical occlusion of a major artery (the middle cerebral artery); very few have used thrombus or hemorrhage or indeed studied animals with spontaneous stroke. A further factor which may have influenced the apparent discrepancy between rodent studies and clinical trials may be that unsurprisingly, stroke has been considered as a neurological disorder given that the clinical outcomes are neurological. Yet stroke is profoundly influenced, and perhaps even caused, by systemic factors, including atherosclerosis, impaired cardiac function, systemic inflammation, and infection.

It is clearly not feasible, or probably helpful, to fully mimic all the common clinical factors associated with stroke simultaneously in animals. To develop aged, atherosclerotic animals, some of which have diabetes, many hypertension and obesity, some would be smokers, have a poor diet and others would have many other confounding factors and comorbidities, as well as exposure to a whole array of potential medicines, would make animal studies as complex and expensive as clinical trials. It would also make interpretation of data near impossible. Animal research, in most countries, is further constrained by legislative and welfare considerations which quite rightly aim to limit suffering.

Thus, we focus here on the major confounding factors known to cause or exacerbate stroke i.e. hypertension, diabetes, obesity, infection, inflammation, atherosclerosis, and age and attempt to review available methods, their robustness and limitations, and available data.

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## 2. Hypertension

Hypertension is the single most important modifiable risk factor (6–9), which increases stroke risk by 20–30% for a 10 mmHg increase in arterial blood pressure (8) and doubles the lifetime risk in individuals with blood pressure above 120/80 mmHg (10).

Hypertension is evident in >50% of stroke patients (6–9). Improved diabetic control (11) and blood pressure reduction (12) both reduce stroke risk.

There are many animal paradigms of hypertension but only a few have been used to study stroke. To date, no stroke studies appear to have been undertaken in hypertensive transgenic rats expressing an extra copy of the renin gene (13) or in the New Zealand, Milan hypertensive or Lyon hypertensive rats (14–16). Hypertension and stroke have been induced in Cynomolgus monkeys by surgical coarctation in animals fed an atherogenic diet (17), but this would not seem to be a practical model for widespread use.

The most widely used hypertensive animals in stroke research are the spontaneous hypertensive rat (SHR) and the stroke-prone SHR (spSHR); the latter was derived by inbreeding of the offspring of SHRs that died of stroke. Over 80% of the spSHR population develops stroke, which is characterized by multifocal microvascular and spongy-cystic parenchymal lesions (18). Wistar Kyoto rats were the parent strain for both (19).

In spSHRs, a high salt diet precipitates renal injury, marked hypertension (>240 mmHg) and rapid onset of hemorrhagic stroke (20, 21), and ~70% of strokes are in the gray matter of the cortex (22). While the number of anastomotic connections between anterior and middle cerebral artery territories in the cortex is similar to WKY rats, their luminal diameter is reduced (23–25), and capacity for vasodilation impaired (26–28).

SHR rats become hypertensive within 2–4 months of birth and attain a stable systolic blood pressure (~200 mmHg) by 6 months. Salt sensitivity is not a major component of their phenotype (29). The hypertension is accompanied by enlarged ventricles (30, 31), cerebral artery smooth muscle hypertrophy (32), increased macrophage activity (33), and reduced body and brain weight (30, 34, 35). Losses after thread occlusion of the MCA are substantially lower (36) than reported for the Dahl salt-sensitive rat.

In both the SHR and spSHR, focal cerebral ischemia leads to greater infarct volumes and behavioral deficits than in normotensive controls and less variability (36–39). The reduced variability is also evident when submaximal infarcts are induced (Howells, personal observation) indicating that this, and the greater propensity to damage, results from reduced cortical collateral flow (40) imposed because of reduced luminal diameter (23–25), and reduced capacity for vasodilation (26–28). Reduced variability is not imposed by the absence of the posterior communicating arteries (41), the mechanism proposed for similar large infarcts, and small variability in the gerbil (42).

We were unable to find evidence of studies of most neuroprotective agents in hypertensive or diabetic animals. For six drugs that have been the subject of systematic review and meta-analysis,

only 10% of the publications identified included the modeling with high blood pressure or diabetes (43). Nicotinamide and FK506 were substantially less effective in animals with diabetes or hypertension (44, 45). NXY-059 was less effective in SHR than in healthy animals (17.6% versus 47.8%;  $P < 0.001$ ) (5) and tPA provided no benefit in SHR but did increase the risk of hemorrhage (Sena and Howells, unpublished data). Hypothermia, however, was slightly more effective in SHR than in SD and Wistar rats (43).

The impact of other neuroprotective agents in SHR rats has not been assessed systematically. Long-term control of hypertension with calcium or AT1 antagonists before MCAo appears to reduce infarction (46–49) while L-arginine has no effect when local CBF is unaltered (50) but reduces infarct volume when accompanied by increased cerebral blood flow (51). MK-801, the archetypal neuroprotectant has been reported to either have no effect (52) or to be less effective in hypertensive than in normotensive controls (53).

Renovascular hypertension can be induced by clipping one renal artery (keeping the kidneys in place, 2 Kidneys, 1 Clip) to produce mild but relatively unstable hypertension (54) that nevertheless exacerbates ischemic injury (55). Clipping both renal arteries in Sprague-Dawley rats leads to stable hypertension with spontaneous stroke characterized by a mixture of small infarcts with thrombotic occlusion and hemorrhagic lesions caused by bleeding from the arteriolar wall in 62% of the animals within 40 weeks (56). Development of spontaneous T2-weighted MRI lesions in these models is tightly dependent upon blood pressure. After partial occlusion of both renal arteries, consistent brain lesions were seen only when blood pressure exceeded 276 mmHg (57). In agreement with these data is the observation that decompressive craniectomy reduces MCAo induced mortality in renovascular hypertensive rats by ~85% (58).

The Dahl salt-sensitive rat rapidly develops marked hypertension (~200 mmHg) when fed a high salt diet (8% NaCl), and this leads to blood–brain barrier disruption, stroke, and death (59, 60). At lower salt concentrations (e.g., 1% NaCl), the same end is reached but the animals survive at least until ~5 months of age (61). Thread occlusion of the MCA after 5 weeks on a high salt diet leads to excessive death or hemorrhage at the MCA/ICA bifurcation. When the MCA was occluded for 120 min, 80% of the animals died or suffered hemorrhage within 24 h, 90 min occlusion still left 40% of the animals with hemorrhage (62).

Like the spSHR and renovascular models, rats made hypertensive by treatment with mineralocorticoid receptor agonist deoxycorticosterone plus salt also suffer spontaneous strokes (63), but they are more readily protected against the effects of acute middle cerebral artery occlusion by antihypertensive treatment with agents such as captopril than by spSHRs (64).

Spontaneous stroke and worsening of the effects of MCAo in these animals may be mediated by stiffening and narrowing of the middle cerebral artery (65).

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### 3. Diabetes/ Hyperglycemia

Diabetes (type 1 & 2) or acute hyperglycemia is evident in 25% (66) and 40% (67) of stroke patients respectively, and both are associated with poor outcome (68–71).

Diabetes can be induced by selectively poisoning pancreatic  $\beta$ -cells with streptozotocin or Alloxan, by selective breeding in the nonobese diabetic (NOD) mouse, the BioBreeding (BB) rat, and occurs spontaneously in the Zucker diabetic fatty rat (72) and the Goto-Kakizaki rat (73). Specific defects in the db gene on mouse chromosome 4 and the fa gene on rat chromosome 5 both lead to leptin receptor defects (74, 75). Isolated hyperglycemia can be induced by infusion of glucose or dextrose (72).

Hyperglycemia accelerates and extends infarct development after MCAo in rats (76), cats (77), dogs (78), and rabbits (79). In cats (77), the three- to fourfold increase in infarct size due to hyperglycemia after permanent middle cerebral artery occlusion was associated with increased death due to edema upon reperfusion. In monkeys, infusing glucose prior to cardiac arrest causes seizures and muscle twitching due to mild edema and necrosis of the cortex and basal ganglia (80). Some authors have reported that the effects of hyperglycemia on infarction are confined to the cerebral cortex (81), while others report that only very early and short-term hyperglycemia may be needed to cause injury (82, 83).

Blood–brain barrier damage and edema (84–87) together with faster and larger infarct development after transient and permanent ischemic lesions (85) are also features of diabetes induced by chemical poisoning of pancreatic  $\beta$ -cells.

Where direct comparisons have been made between transient hyperglycemia and diabetes, the data are conflicting; some report similar degrees of exacerbation of postischemic injury (48) or that diabetes induces significantly more injury (88). Interestingly, when acute hyperglycemia lessens the impact of tPA therapy (83, 89), it has no effect on endogenous tPA expression, while a similar but persistent elevation of blood glucose (~15 mmol/L) in streptozotocin-treated rats leads to a complete depletion of tPA protein and >6-fold loss of tPA mRNA expression (88).

In the BioBreeding (BB) rat, the effects of hyperglycemia on ischemia are gender and region specific. Males and females exhibit similar degrees of cortical injury, but subcortical infarction is larger in males (90). Similar observations have been made in the db/db mouse, where female diabetic mice became more hyperglycemic

and acidotic than the males even though they were more resistant to ischemic damage (91).

The recently described Goto-Kakizaki rat, which develops mild hyperglycemia at 6 weeks of age, is unusual in producing smaller infarcts after extended (3 h) MCAo (but high rates of subcortical hemorrhagic transformation) than do nondiabetic controls (73). While this has been interpreted as the result of diabetes-induced vascular remodeling (73), the infarcts look like the hypothalamic lesions produced when only hypothalamic perforating arteries are occluded (42) suggesting that reduced effectiveness of thread occlusion might be a sensible alternative explanation. The observation that injury caused by compression-induced cortical ischemia is greater in Goto-Kakizaki rats than in healthy Wistar controls (92) supports this hypothesis.

The impact of diabetes on efficacy of neuroprotection is not clear. Using insulin to control hyperglycemia has been reported to return neural injury to control levels and offer marked neuroprotection if blood glucose is reduced to below normal (93, 94). Similar protective effects on infarction were reported when insulin was used together with tPA in normoglycemic animals to treat thromboembolic strokes. However, others report that tight glycemic control does not improve infarct size in male BB rats (90) and that, despite reduced infarct volumes, mortality was as high after insulin treatment alone (47%) as it was when combined with tPA (38%) (95).

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## 4. Obesity

### **4.1. Association with Stroke Risk**

Growing evidence suggests that obesity predisposes to cardiovascular disease including stroke (96–99). This association may be mediated indirectly via the susceptibility of obese individuals to established stroke risk factors, such as atherosclerosis, hypertension, hyperlipidemia, and insulin resistance/diabetes (100). In addition, there are considerable data indicating an independent association between obesity and stroke. Some studies have reported a graded elevation in stroke risk for each unit increase in body mass index (BMI) greater than 25 (97, 99) although others have not observed such a close correlation (101, 102). More recently, the validity of BMI as the optimal index for assessing the impact of obesity on cardiovascular disease has been questioned (103). Abdominal adiposity as measured by waist-to-hip ratio may provide better estimates of the health risks associated with obesity (104, 105). In support of this, a recent study demonstrated a significant and graded association between the risk of stroke and transient ischemic attack and markers of abdominal obesity, including waist-to-hip ratio that was independent of hypertension and diabetes (103).

#### **4.2. Animal Models for the Study of Interactions Between Obesity and Stroke**

A huge number of animal models of obesity have been reported although the extent of characterization for each of these varies widely (106). In view of the predominance of rodent models in experimental stroke studies, obesity in mice and rats probably offers the greatest utility for experimental stroke researchers. Rodent models of obesity may arise through genetic mutation (spontaneous or targeted) or may be induced by some type of environmental stimulus e.g. high-fat diet (106). Genetic models may further be subdivided as monogenic and polygenic. Spontaneous mutants giving rise to monogenic rodent models of obesity are the most commonly used and have been characterized most extensively. Due to the diversity of models available, multiple factors deserve consideration when choosing an obese model to incorporate in experimental stroke studies. These include strain, method of induction, sex-dependency, age of onset, severity and associated phenotypes. The following sections summarize some of the benefits and limitations/constraints of the most commonly used rodent obese models that are likely to be most worthy of consideration for experimental stroke studies. Readers are referred to these excellent resources for detailed accounts of these and other obesity models (106–108).

##### **4.2.1. *ob/ob* Mice**

*ob/ob* Mice carry a spontaneous mutation in the *Lep* gene encoding the leptin protein which is abundantly produced by adipocytes (109). Mice homozygous for the *Lep<sup>ob</sup>* mutation are deficient in leptin, which is an appetite-suppressing hormone. As a result, *ob/ob* mice (on Bl/6 background) are hyperphagic, and are first recognizable as obese at 4 weeks of age when fed a normal diet. Adult *ob/ob* mice may become three times the weight of wild-type controls. They are glucose intolerant, insulin resistant and hyperinsulinemic but are not hyperglycemic except during a short period in early adulthood (subsides by 12–16 weeks) (107, 108). Our own studies (McColl, Lawrence, unpublished) indicate a marked increase in hemorrhage on *ob/ob* mice exposed to MCAo.

##### **4.2.2. *db/db* Mice**

*db/db* Mice carry a spontaneous mutation in the leptin receptor gene (*Lepr*), therefore there is a similar defect in the leptin axis as observed in *ob/ob* mice (110). Although obese, male mice homozygous for the *Lepr<sup>db</sup>* mutation (on Bl/6J background) are most commonly used as a model of diabetes (or obesity with diabetes) (111). These mice are also insulin resistant, hyperinsulinemic, and hyperlipidemic. Furthermore, and in contrast to *ob/ob* mice, they develop severe hyperglycemia and show symptoms consistent with overt diabetes (107).

##### **4.2.3. Zucker (*fa/fa*) Rats**

The “fatty” Zucker rat has been extensively used in studies of obesity. Similar to the *db/db* mouse, the Zucker rat has a spontaneous



mutation in the leptin receptor (*fa* mutation) causing defective leptin signaling (112). Homozygous (*fa/fa*) mutants develop obesity and become hyperlipidemic and moderately insulin resistant (112). In contrast to *db/db* mice, however, Zucker rats do not develop overt diabetes (107, 112).

#### 4.2.4. Corpulent (*cp/cp*) Rats

Corpulent rats carry the spontaneously arising corpulent (*cp*) mutation, another mutation, affecting the leptin receptor rendering it dysfunctional. Rats homozygous for the mutation (*cp/cp*) develop obesity, insulin resistance, hyperinsulinemia, and hyperlipidemia but do not progress to type 2 diabetes (107). The original hypertensive mutant strain (designated SHROB) was backcrossed to SHR, WKY, or LA strains, thus generating SHR/N-*cp* (hypertensive), WKY/N-*cp* (normotensive control for SHR/N-*cp*) and LA/N-*cp* (normotensive) models (107). A further strain designated JCR:LA-*cp* was developed as an outbred colony from early LA/N-*cp* breeding stock. Significantly, male JCR:LA-*cp* rats homozygous for the *cp* mutation develop spontaneous vascular pathology that includes atherosclerotic lesions similar to those observed in humans and ischemic lesions in the heart (113).

#### 4.2.5. Diet-Induced Obesity

Several different types of dietary regimens have been used to induce obesity in rodents, including high-fat diets, high-energy diets (moderately high fat and high sugar), and palatable liquid diets. DIO has been characterized in both rats and mice, although there are strain-specific differences in susceptibility in both species. Sprague-Dawley and Long Evans rats and C57Bl6/J mice are well established as susceptible strains, with males particularly predisposed (114, 115). Hyperphagia, obesity, insulin resistance, hyperinsulinemia, glucose intolerance, and hyperlipidemia are common features on adjusted diets, although the relative severity of each of these will depend on the exact composition of diet and strain susceptibility (116, 117). A commonly employed diet in male C57Bl6/J incorporates a 45% or 60% (by energy) fat component, with controls being fed 10% fat. Mice are typically maintained on diets for up to 20 weeks. Modified diets such as these are available commercially (e.g., from Research Diets Inc.).

### 4.3. General Considerations When Selecting Obesity Models for Experimental Stroke Studies

The animals described above illustrate some of the more commonly used rodent obesity models that may be applicable to incorporate in experimental stroke studies. The optimal model for any study will depend on multiple factors, including the experimental hypothesis under testing, confounding factors, purchase and maintenance costs of animals/diets and stroke model used.

All the monogenic spontaneous mutant animals outlined above arise from deficiencies in the leptin/leptin receptor signaling axis. Leptin has a diverse array of actions in addition to its role

in appetite regulation, including the effects on the immune and cardiovascular systems (83, 118). Accordingly, it could not be excluded that any effects on stroke responses in these models are because of leptin signaling insufficiency independent of obesity. To counter this, leptin replacement groups could be included although these are unlikely to fully restore the leptin axis to normal physiological conditions. This highlights a relative advantage of the diet-induced models. Furthermore, DIO is a more realistic model of the dietary component that is a key contributor to human obesity.

In humans, the vascular consequences commonly accompany obesity and metabolic syndrome that are of most relevance to stroke, since these are likely to mediate the link between metabolic disturbances and stroke susceptibility. However, with the exception of the JCR:LA-*cp* strain, all obese models are resistant to the development of overt human-like vascular pathology (107). For this reason, the JCR:LA-*cp* model that perhaps most closely resembles the metabolic syndrome associated with obesity most likely to present in stroke patients i.e. with downstream vascular abnormalities.

Hyperglycemia and diabetes are associated with poorer outcome in stroke patients (see above); therefore, it may also be desirable to induce obesity without affecting glycemic status. This might enable the role of increased adiposity itself to be more easily delineated. This should be possible since some models (e.g., ob/ob mice after 12–16 weeks) are normoglycemic despite being markedly obese.

On a more practical note, it should be considered that obese animals, by definition, have large deposits of adipose tissue and that this has the potential to complicate the surgical induction of stroke (particularly filament models where access to the carotid vessels is a prerequisite). However, our own experience suggests that despite marginally extended surgery times, subcutaneous adiposity does not unduly affect success rates. This is also borne out by the recent studies that have emerged combining experimental obesity and stroke (119–122). All such studies have found that the severity of brain damage is markedly increased in obese rodents compared to corresponding wild-types (see below). This suggests that the basal severity of the stroke model may need to be titrated (e.g., by adjusting occlusion duration) to avoid excessive mortality and morbidity in the obese experimental group. Experimental stroke is commonly induced in 10–12-week-old rodents. The rapid onset of obesity in the monogenic models enables similar aged animals to be used. In contrast, DIO involves long durations of feeding adjusted diet (perhaps up to 20 weeks); therefore, the age at which stroke can be induced in these animals is likely to be significantly older. Thus, the age and also the cost of long-term dietary modification and animal housing are important to consider. A further consideration is the difficulty in determining the doses of

any pharmacological intervention. Given the very different body weights, it is not possible to give interventions on a dose per unit body weight basis and highly lipophilic substances may be preferentially taken up into adipose tissue.

These are just some of the many important factors to consider when selecting an obesity model to incorporate in experimental stroke studies. With good experimental planning and design and with particular attention to potentially confounding variables, most models are likely to enable a better understanding of the impact of obesity on stroke.

#### **4.4. Overview of Experimental Obesity–Cerebral Ischemia Studies**

It is only in the last 2 years that studies investigating the impact of obesity on experimental stroke in rodents have emerged ([119–122](#)). All studies have found significantly increased ischemic brain damage in the obese group (see Table).

Author	Model	% Change in infarct	References
Nagai et al.	ob/ob 15 wk 42% high-fat diet	30% ↑ 30% ↑	( <a href="#">119</a> )
Terao et al.	ob/ob	75% ↑	( <a href="#">121</a> )
Mayanagi et al.	ob/ob	43% ↑	( <a href="#">122</a> )
Osmond et al.	Zucker (fa/fa)	170% ↑	( <a href="#">120</a> )

## **5. Atherosclerosis**

The significance of atherosclerosis in the context of stroke risk is highlighted by the estimation that thromboembolism of atherosclerotic origin is the principal etiological factor in approximately 50% of stroke patients ([123](#)). These statistics suggest that incorporating atherosclerosis in at least some preclinical stroke models should be a priority and should help to more accurately recapitulate important features of clinical stroke.

### **5.1. Animal Models of Atherosclerosis**

An extensive range of animal species have been used to study atherosclerosis, including large animals such as nonhuman primates, pigs, rabbits, and, more recently, mice. The majority of experimental stroke studies are performed in rodents therefore murine models of atherosclerosis are likely to offer the greatest utility for experimental stroke researchers.

The mouse is naturally resistant to spontaneous atherosclerosis, which may in part be the result of the HDL-dominant and

antiatherogenic lipid profile in mice compared to the atherogenic LDL-dominant profile in humans (124). For this reason, feeding an atherogenic (commonly high-fat, high cholesterol) diet to genetically modified mice with enhanced susceptibility to atherosclerosis forms the basis of most currently used atherosclerosis protocols. A variety of diets have been described and the reader is referred to these excellent articles for more detailed information (124, 125).

#### 5.1.1. *C57Bl/6J*

Most wild-type mice are resistant to atherosclerosis when fed a conventional rodent chow diet; therefore, aggressive dietary manipulation is necessary to induce lesion formation. When fed a high-fat (30%), high cholesterol (5%), cholate-containing (2%) diet, or the “Paigen” diet (15% fat, 1.5% cholesterol, 0.5% cholate), C57Bl/6J mice develop small aortic lesions resembling the fatty streaks in humans by 4–5 months of age (124, 125). However, the lesions rarely progress beyond the early foam cell/fatty streak stage which represents the major limitation of these models.

#### 5.1.2. *ApoE<sup>-/-</sup> Mice*

In order to induce advanced atherosclerosis in mice, it is necessary to use genetically-modified, susceptible strains, most commonly in combination with dietary modification. The most frequently used and best characterized strains are the apolipoprotein E (apoE)<sup>-/-</sup> and low density lipoprotein receptor (LDLR)<sup>-/-</sup> mice. ApoE is a glycoprotein synthesized in many tissues and at highest levels in the liver and brain. It is a structural component of lipoprotein particles and acts as a ligand for receptors such as LDLR that mediate cellular internalization of apoE-containing particles (126). ApoE<sup>-/-</sup> mice when fed a chow diet are hypercholesterolemic and significantly have human-like lipoprotein profiles (i.e. low HDL) (127, 128). These mice develop lesions throughout the macrovasculature (but not in cerebral vessels) that progress to advanced plaques with a well-defined fibrous cap consisting of smooth muscle cells, extracellular matrix and foam cells (124). Feeding a Paigen diet or the more physiological “Western” diet (21% fat, 0.15% cholesterol, cholate-free) accelerates the development of lesions at all stages (129).

#### 5.1.3. *LDLR<sup>-/-</sup>*

LDLR is the principal receptor mediating the uptake of LDL via binding of its major ligand apoB (130). LDLR<sup>-/-</sup> mice develop more modest lipid abnormalities relative to apoE<sup>-/-</sup> mice, and therefore are much more resistant to atherosclerosis on a low-fat chow diet. However, when fed the Paigen or Western diets, LDLR<sup>-/-</sup> can develop intermediate-advanced lesions affecting multiple parts of the vasculature after 2–3 months (125).

**5.2. General  
Considerations  
When Choosing  
Atherosclerosis Models  
for Experimental  
Stroke Studies**

In view of the various models of atherosclerosis available, several factors are likely to be worthy of consideration when selecting a model to incorporate in experimental stroke studies. The similarity of the model to the human condition is of prime concern. In the context of atherosclerosis, the composition of the vascular lesions and the lipid profile are particularly important issues. As described above, the vascular pathology and lipid profiles observed in apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice fed modified diets most closely reflect the human disease. Another advantage of these models is that the progression to advanced lesions progresses fairly rapidly, thereby reducing the duration of experiments and associated costs in maintenance. However, the use of genetically-modified mice in combination with dietary modification also introduces potentially confounding (124) variables. For example, there is evidence that the diets themselves may be proinflammatory and thus it may be difficult to distinguish between effects caused by atherosclerosis itself rather than diet alone. Analogous to this, genetic deficiency in apoE or LDLR may have important consequences independent of the induction of atherosclerosis. Notably, apoE has a number of important activities in the CNS, including homeostatic lipid transport (131). In addition, apoE has several neuroprotective and neuroregenerative features (131). For example, the extent of brain damage is significantly greater in apoE<sup>-/-</sup> mice (on regular chow diet) after a variety of acute brain injuries, including focal and global cerebral ischemia (131). Anti-inflammatory, antioxidant, and antiexcitotoxic properties have all been described for apoE in the brain (132). Although LDLR is a major receptor for apoE in peripheral tissues, this is not the case in the brain, where receptors such as LDLR-like protein (LRP) and apoE receptor 2 (apoER2) are the predominant transducers of apoE signaling (133). The use of LDLR<sup>-/-</sup> mice may therefore avoid some of the complications associated with apoE<sup>-/-</sup> mice since CNS apoE signaling pathways may be less severely compromised. Regardless, potential complications in interpreting results from studies combining genetic and/or dietary models of atherosclerosis with experimental stroke will require the inclusion of multiple strain and diet control groups. If these are adequate, then it should be possible to extract meaningful conclusions from such studies. Aside from these design issues, an important practical consideration relates to the potential disruption of atherosclerotic lesions if stroke is induced by filament advancement along the internal carotid artery. Although a full characterization of vascular pathology in the carotid and intracranial arteries is lacking, it is conceivable that filament advancement could dislodge atheromatous material, thus generating emboli-like particles which could complicate occlusion uniformity.

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## 6. Aging

Since the incidence of stroke is higher in the elderly population compared to young people, it may be tempting to hypothesize that aging per se could facilitate stroke occurrence and/or impair outcome. It is, however, very difficult to separate the effect of age itself from many of its frequent peripheral or central comorbidities such as obesity, diabetes, hypertension, arteriosclerosis, or the presence of microhemorrhages and senile plaques in the brain. The use of aged animals (especially rodents) in experimental models of stroke may not perfectly reflect the human situation due to life span, strain and gender differences, and many other confounding factors. Nevertheless, several data have indicated similar effects of aging regarding infarct size, maturation, vascular and neuronal damage between patients and experimental animals.

It is known that in humans, cerebral blood vessels undergo profound changes with aging, including reduced capillary density and the formation of thickened and fibrotic basement membranes. Endothelial cells exhibit a reduced number of mitochondria, and in pericytes (which normally replace smooth muscle cells) degenerative processes are seen. In the aging human cortex, microhemorrhages become frequent, identified by their content of hem, red blood cells, collagen and clotting factors, and their spatial relationship to capillaries (134, 135). Similarly, vascular density, arteriole–arteriole anastomoses and basal blood flow decreases with age in rats (136), and structural changes are seen in basal lamina and endothelial cells (137).

Experimental stroke in rats generally (although not uniformly) reveals many factors which are altered in aged animals. In aged rats, increased mortality rate, but similar recovery and infarct volume were found (138). Another study showed that aged rats suffer larger infarctions, reduced functional recovery and increased BBB disruption that precede observable neuronal injury (139). Aging has been shown to reduce hypoxia-induced microvascular growth in the rodent hippocampus (140). Basal neurogenesis is impaired in the subgranular and subventricular zones of aged animals, although the magnitude of striatal neurogenesis after stroke is similar in young and old animals (141). Spontaneously hypertensive rat (SHR) models revealed larger infarcts in aged (18–22 months old) animals after photothrombotic distal MCAo compared to adults (142), and ischemic neuronal damage in the hippocampus and striatum was produced by 20 min of transient global ischemia in aged but not in adult rats (143).

In a recent report, stroke damage was found to increase with aging in female mice, whereas male mice had decreased damage after MCAo, which correlated with blood–brain barrier

permeability changes. Interestingly, edema formation was less profound in aging mice, independent of sex and extent of histologic damage (144).

These examples may indicate that depending on the ischemic model used, species, strain and gender differences, the observable effect of aging can be largely different. This may require much more comprehensive studies in the future to understand the key elements of the process of aging on damage formation, inflammation, and recovery.

### **6.1. Inflammation and Infection: Risk Factors and Disease Consequences**

To satisfactorily model inflammation and infection as risk factors in experimental stroke, it is important to understand the initiation, maintenance, and termination of both central and peripheral inflammatory changes, with special respect to their interactions in cerebral ischemia. These processes can occur simultaneously in stroke, but the root causes may have started decades before their *consequences* are observed in the clinical practice, hence this area of experimental research is fraught with confounding factors.

Inflammation is a highly complex and normally well-coordinated biological response of vascular tissues to harmful stimuli. Typically, in peripheral tissues, acute inflammation induced by pathogen invasion or tissue injury includes a sequence of events aimed at eliminating deleterious agents and helping wound healing processes. Several elements of this reaction contain partially stimulus-specific components (for example the activation of certain toll-like receptors, which can recognize given pathogen-associated patterns) and also many common features, such as dilation of local blood vessels, the activation, recruitment of leukocytes and release a wide range of active peptide and nonpeptide mediators in inflamed tissues. While uncontrolled or prolonged inflammation can be highly detrimental in the periphery, its consequences may even be more serious in the brain, where anti-inflammatory and reparative processes would never result in complete recovery because of the very limited replacement of damaged neurons.

Many elements of cerebral ischemia-induced inflammatory changes in the brain are very similar to the general pattern of the peripheral inflammatory response. The early central inflammatory events include the production of reactive oxygen species (nitric oxide, superoxide) expression of proteolytic enzymes (MMP-9, MMP-2), proinflammatory cytokines (IL-1 $\beta$ , TNF $\alpha$ , IL-6), chemokines (CINC-1, KC, MCP-1, MIP-1 $\alpha$ ), and vascular adhesion molecules (ICAM-1, P-selectin, I-selectin). Activation of resident glial cells (astroglia, microglia) and early infiltration of granulocytes followed by monocytes, macrophages, and other hematogenous cells is observed within hours to weeks after experimental stroke (58, 115, 121, 145–148).

Inhibition of inflammation is protective in most experimental models of stroke.



Because central inflammatory processes in stroke are associated with the disruption of the BBB and the formation of the ischemic damage, it is not easy to decide whether these changes are consequences of the cerebral ischemic event itself or indeed contribute to damage formation. This may be one of the reasons why stroke has long been considered as a neurological disorder and the potential effect of systemic factors was largely neglected. In line with this, several studies that showed neuroprotection after cerebral ischemia by inhibiting certain components of the inflammatory response failed to convincingly demonstrate whether this effect was centrally or peripherally mediated. In fact, inhibition or deletion of proinflammatory cytokines (149, 150), chemokines (151, 152), chemokine receptors (153, 154), treatment with tetracycline derivatives (155), etc., have all been shown to be protective in experimental rodent models of focal cerebral ischemia. One can also ask why such a robust inflammatory response is initiated in response to cerebral ischemia if it is harmful to the organism. Does postischemic inflammation involve similar mechanisms and/or effects in the periphery and in the brain? Although several reports showed that oxidative stress and recruitment of inflammatory cells are harmful mainly in stroke, superoxide-initiated inflammation and monocyte recruitment seem crucial for peripheral, flow- or ischemia mediated vascular remodeling (156, 157). May modest and well-controlled inflammation facilitate processes of repair? For example, inhibition of matrix metalloproteinases in several rat or mouse MCAo models reduced damage size, brain edema formation, neuronal apoptosis (136, 158, 159), but facilitated cell death in intracerebral hemorrhage (160) or inhibited neuronal progenitor cell migration after photothrombotic ischemia in mouse (161). These examples indicate that the development of more appropriate animal models, tissue- and cell-specific deletion, inhibition or overexpression of inflammatory mediators will be essential for a deeper understanding of underlying inflammatory mechanisms in stroke.

Inflammatory processes may differ between species and strains because of the differences in peripheral leukocyte populations, cerebral vasculature, size of the brain, kinetics of aging, etc.; therefore, in the future it may be advantageous to study cerebral ischemia more frequently in large mammals such as sheep (162) or swine (163). However, the availability and cost of animals may limit the number of these kinds of experiments.

## **6.2. Systemic Inflammation and Infection: The Need for Appropriate Experimental Models**

Inflammation is elicited as a defense mechanism against infectious agents but is not necessarily caused by infection. The co-occurrence of these factors and the large overlap between the expression of infection-induced and ischemia-induced mediators create many confounding effects in clinical practice and in experimental stroke research. Pre-existing infection or systemic inflammation



may induce and is very likely to affect cerebral ischemia and relevant experimental models are crucial to understand the underlying mechanisms. In patients, acute- and chronic infection can be a trigger of stroke, and preceding infection worsens stroke outcome. Furthermore, poststroke infections affect clinical outcome (164). Respiratory- and urinary tract infections are the most common types of infection preceding stroke after adjustment for age and conventional vascular risk factors (165, 166).

To date, the effect of systemic inflammation and infection on experimental cerebral ischemia has been largely ignored in experimental research. Similarly, the effect of cerebral ischemia on the induction or modulation of peripheral inflammatory processes has also been overlooked. It is now established that cerebral ischemia itself induces a rapid, but often prolonged peripheral inflammatory response. The level of proinflammatory cytokines, such as IL-6, IFN $\gamma$ , and MCP-1, becomes elevated in the plasma within hours and may remain elevated for several days in stroke patients and for 1–3 days in animals with experimental stroke. This is accompanied by the induction of chemokines and chemokine receptors in peripheral organs; long-term elevation of acute phase proteins and white blood cell counts in the circulation (167, 168). It is reasonable to ask how these processes are mediated and what the consequences of the activation of the peripheral inflammatory response on central inflammatory changes in stroke are.

It is crucial to reveal the mechanisms of peripheral inflammatory changes in ischemic damage formation and to understand how a pre-existing inflammatory event can modulate all these processes in different experimental models. For example, depletion of neutrophils in the periphery showed that these cells are a major source of oxygen radicals during reperfusion after focal cerebral ischemia in the rat MCAo model (169). Systemic inflammatory stimulus induced by intraperitoneal lipopolysaccharide (LPS) or IL-1 $\beta$  administration exacerbates brain damage via a neutrophil-dependent mechanism in a mouse MCAo (170) and alters the kinetics of cerebrovascular tight junction disruption through neutrophil-derived MMP-9 (171). Therefore, studies using young, healthy animals with no additional inflammatory diseases may underestimate the impact that systemic factors have on patients with multiple comorbidities.

Another aspect of the peripheral changes caused by cerebral ischemia is profound immunosuppression, both in patients and in experimental animals. These changes include splenic atrophy, reduced B- and T cell counts and proliferation, reduced proinflammatory protein expression by blood cells (168, 172, 173). The underlying mechanisms may be similar to those leading to systemic immunodepression after brain trauma (174). It is not surprising that stroke-associated pneumonia is an important independent contributor to poor outcome in patients (175, 176).

Experimental rodent models have been established to reveal the potential mechanisms and to find therapeutic targets. In a mouse model of focal cerebral ischemia, extensive apoptotic loss of lymphocytes, and a shift from T helper cell (Th)1 to Th2 cytokine production were observed and animals developed spontaneous septicemia and pneumonia (177). Preventive antibacterial treatment improved the general medical and neurological outcome (178). It seems, therefore, that several parameters have to be taken into consideration for the experimental design to model the clinical situation because the interaction between ischemic and immune processes seems highly complex.

Due to the large overlap between inflammatory changes induced by ischemia and infection, in complex experimental models it may be very difficult to interpret results and to dissect mechanisms. Several chemokines and proinflammatory cytokines, which become activated after stroke in the brain or in the periphery (167, 168) are expressed in response to viral (179) and systemic bacterial infections (180). In addition, pathogen detecting sensors such as toll-like receptors (TLRs), and NOD-like receptors, activated by viral- (179) or microbial-derived molecules (181), not only recognize various self-ligands such as heat shock proteins (182) or extracellular matrix components (159), but also function as endogenous damage sensors, becoming activated during neuroinflammation and stroke (183, 184) and play a role in the production of cytokines such as interleukin-1 beta (179). In line with this, mice lacking either functional TLR4 (185) or TLR2 (186) are less susceptible to transient focal cerebral ischemia and reperfusion damage. Unfortunately, only very few studies have been performed on gnotobiotic animals; continued research could give further insights to the potential effects of the endogenous flora.

Timing can be another confounding factor in modeling the effects of inflammation or infection in experimental stroke research. As discussed above, acute or chronic (systemic) inflammation may worsen the outcome after stroke, but preconditioning with LPS or preactivation of TLRs may be protective through mechanisms similar to ischemic tolerance. These events are most likely mediated through the hyporesponsiveness or reprogramming of TLR-mediated signaling (187, 188).

Inflammation is associated with most known comorbidities. Interestingly, many comorbidities associated with stroke (such as diabetes, atherosclerosis or obesity) involve inflammation. For example, adipose tissue is now recognized as an abundant source of inflammatory mediators, and obesity is now considered a state of chronic inflammation with significantly elevated systemic levels of various cytokines, including tumor necrosis factor- $\alpha$  and interleukin-6. Inflammation is strongly implicated in the development and/or progression of insulin resistance/diabetes, hypertension, and vascular disease-themselves key factors increasing stroke

susceptibility in obese individuals (189–191). Markers indicative of an increased prothrombotic tendency (e.g., increased levels of von Willebrand factor, plasminogen activator inhibitor-1) are also evident in obese individuals (191, 192). Inflammatory mediators such as TNF- $\alpha$  promote procoagulatory mechanisms and inhibit antithrombotic mediators (193), suggesting that increased inflammation in obesity could cause a shift from anti- to prothrombotic status, thus favoring the development of a “stroke-prone” state. Similarly, atherosclerosis is a chronic inflammatory disorder that primarily affects large and medium-sized arteries at sites of disturbed blood flow (194). Atherosclerotic lesions consist of endothelial cells, vascular smooth cells, and various immune cells, including T lymphocytes and monocytes and macrophages (195). These cells, in concert with a diverse array of soluble inflammatory mediators, modified lipids and proteases drive the progression of plaques from a relatively benign to highly unstable state prone to rupture. Plaque instability and rupture trigger thrombosis and intravascular occlusion, leading to stroke. The contribution of inflammatory mediators to the initiation or progression of these common comorbid conditions affecting stroke patients suggests that inflammation is a more important contributor to the incidence and consequences of stroke in clinical reality than it is in experiments undertaken to date. It will be important to determine how a pre-existing inflammatory state resulting from acute (e.g., infection) or chronic (e.g., atherosclerosis) inflammatory conditions modifies the peripheral and central inflammatory processes when an ischemic event occurs. Indeed, there is recent experimental evidence that peripheral inflammatory stimuli markedly alter the magnitude and kinetics of the peripheral and neurovascular inflammatory responses to experimental stroke (170, 171).

Beyond providing potential mechanisms for the interaction of systemic infections with stroke, these examples also highlight some of the technical considerations and difficulties involved in experimental models. As peripheral inflammation may modify the expression of inflammatory mediators in the inflamed CNS, cerebral ischemia may increase or blunt infection-induced changes in the periphery. The examination of multiple organs, evaluation in various time-points after ischemia and systems biology approaches can therefore be of great importance for achieving a more complete understanding.

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## 7. Conclusions

Our failure to adequately model the factors which cause a stroke and contribute to outcome in experimental studies may have been a significant factor in the failure of potentially promising

therapeutic interventions. It is clear from laboratory studies that factors such as obesity, diabetes, atherosclerosis, and age have a profound effect on ischemic brain injury in rodents. But their inclusion in experimental studies is not without problems. Many of the methods used to induce such conditions have confounding effects. We rarely study spontaneous stroke caused by the factors which are known to predispose humans to stroke, and few studies have assessed the effects of multiple interventions, for example to mimic the cocktail of treatments applied to many patients. In spite of these difficulties, it seems clear that wider use of animal models with comorbidities is important for future research on stroke.

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